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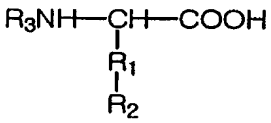
(54) Title: PROCESS FOR THE PREPARATION OF CALCITONIN



(I)

(57) Abstract

There is provided by the invention a process for preparing calcitonin by means of stepwise condensation, and especially the process for preparing calcitonin with peptide fragments obtained by using a side chain protected amino acid derivative of general formula (I) or pharmaceutically acceptable salt thereof, wherein R^1 may represent $-CH_2CH_2CH_2CH_2NH-$, $-CH_2CO-$ or $-CH_2CH_2CO-$, and R^2 may represent phenylthioethyloxycarbonyl(Ptc), phenylsulfonylethyloxycarbonyl(Psc), phenylthioethyloxy(OPte) or phenylsulfonylethyloxy(OPse), and R^3 may represent hydrogen or α–amino protecting group.

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PROCESS FOR THE PREPARATION OF CALCITONIN

TECHNOLOGY OF THE INVENTION

The present invention relates to the peptide synthesis, especially to the process for preparing calcitonin by using new side chain protected amino acids with high purity in good yield.

BACKGROUND OF THE INVENTION

Peptides can be obtained by i) extracting from natural products ii) DNA recombination technique iii) chemical synthesis, among them i) and ii) have the limits, for example the low yield of active extract, the uncertainty of the identification of purity and the difficulty of the removal of impurities from microorgaism. Thus, the chemical synthesis have been widely used for larger scale commercial production of peptides, and in parallel with the advances in the new methodology of it.

In general the chemical synthesis is categorized to classical liquid phase method doing on solution and solid phase method using insoluble polymer. The latter method is not adjustable for mass production of peptides, since it has a few drawbacks particularly; i) side chains of the amino acids which target peptide is composed of have to be fully protected, ii) the process is carried out by using excess of amino acids under limited coupling condition, iii) it is difficult to remove the impurities builded up in big peptides, iv) a coupling can not be unexpectedly completed, probably due to the folding of the growing peptide, v) after completion of the polypeptide chain, cleavage of the resin reduce the quantity of the product deeply, vi) the resins can not be loaded too heavily with the first residue, vii) the resin, amino acid derivatives, reagent, and solvents for solid-phase technique are all quite expensive.

The former method is more likely to be used for the preparation of larger quantities and higher qualities of peptides, since i) side chains of the amino acids can be minimally protected, ii) various coupling methods can be freely choosed, iii) growing peptide can be purified at any step, iv) quantity of the

reactant can be controlled unrestrictly.

However, in the case of long chain peptide, for example residues 25 to 50, the liquid phase method has the limits on preparing the high quality of peptide largely because of i) time-consuming process, ii) possibility of the side reaction, iii) low-efficiency of the reaction due to solubility, iv) difficulty of the purification of final peptide.

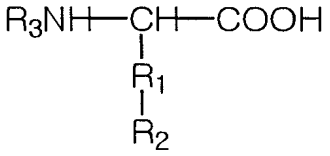
In this point, it is valuable and requisite today to develope the new amino acid derivatives and the process useful for larger scale preparation of peptide with high purity and in good yield based on liquid phase method.

SUMMARY OF THE INVENTION

The invention provides new procedure to prepare high quality of calcitonin efficiently by liquid phase synthesis, combined with conventional segment condensation of the fragments obtained by stepwise coupling with Boc-protected amino acids using coupling agents(DCC, HOBT) or active esters.

Also, the invention provides new methodology for the preparation of peptide using newly developed side chain and C-terminal protecting groups, based on liquid phase synthesis.

The object of the invention is to provide new method for obtaining calcitonin by the use of newly side chain protected amino acid derivatives or the salts having the general formula (I).



(I)

wherein R^1 may represents $-CH_2CH_2CH_2CH_2NH-$, $-CH_2CO-$ or $-CH_2CH_2CO-$, and R^2 may represents phenylthioethyloxycarbonyl(Ptc), phenylsulfonylethyloxycarbonyl(Psc), phenylthioethyloxy(OPte) or phenylsulfonylethyloxy(OPse), and R^3 may represents hydrogen or α-amino

protecting group.

Another object of the invention is to provide new efficient way to prepare large quantities of high quality calcitonin by means of assembly of the following fragments 1 to 7.

Calcitonin may be illustrated as the following formula (II).

H-Cys-A^1-A^2-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His
-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-A^3-A^4-Thr-Gly-A^5-Gly-Thr-Pro-NH_2
(1,7-disulfide bond) (II)

H-A^5-Gly-Thr-Pro-NH_2 (29-32)	Fragment 1
Boc-Arg-A^3-A^4-Thr-Gly-OH (24-28)	Fragment 2
Boc-Leu-Gln-Thr-Tyr-Pro-OH (19-23)	Fragment 3
Boc-Leu-His-Lys(X^1)-OH (16-18)	Fragment 4
Boc-Lys(X^1)-Leu-Ser-Gln-Glu(X^2)-OH (11-15)	Fragment 5
H-Ser-Thr-Cys(X^3)-Val-Leu-Gly-OY TFA (5-10)	Fragment 6
Boc-Cys(X^3)-A^1-A^2-Leu-OH(1-4)	Fragment 7

wherein A^1 may represents Ala or Ser, and A^2 may represents Asn or Ser, and A^3 may represents Asn or Asp(OPse)(wherein Pse is phenylsulfonylethyl), and A^4 may represents Thr or Val, and A^5 may represents Ala or Ser; X^1 may represents phenylsulfonylethoxycarbonyl(Psc), Benzyloxycarbonyl(Z), or fluorenylmethoxycarbonyl(Fmoc) group for blocking of the side amino function of lysine, and X^2 may represents phenylsulfonylethyl(Pse), t-butyl, benzyl, or p-nitrobenzyl group for blocking of the side carboxyl function of glutamic acid, and X^3 may represents acetamidomethyl(Acm) or trityl(Trt) group for blocking of the thiol function of cysteine; Y may represents p-nitrophenylsulfonylethyl(Nse), phenylsulfonylethyl(Pse), benzyl or ethyl group for blocking of C-terminal.

DESCRIPTION OF THE INVENTION

The invention is based on the strategy of segment condensation which has obvious advantages in the synthesis of longer peptide chain. This methodology, however, has the likely racemization of the activated C-terminal residue of the carboxyl component. To reduce the degree of racemization, following methods are recommended; i) good selection of the activated carboxyl residue: glycine and proline are favorable, ii) controle of the reaction condition: low polarity solvent, neutral pH, low temperature, iii) appropriate selection of the mothod for activating the carboxylic acid component, iv) use of α-alkoxycarbonyl protected amino acids in the coupling reactions.

The invention is intended to give the efficient production of calcitonin by the process, considering the points mentioned above. In accordance with the invention, there is provided the methodology useful for larger scale synthesis of calcitonin.

To clarify the superiority of the invention by an embodiment, the process for preparing salmon calcitonin described below and newly developed amino acid derivatives will now be explained.

H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2(1,7-disulfide bond) (salmon calcitonin)

H-Ser-Gly-Thr-Pro-NH_2(29-32)	Fragment 11
Boc-Arg-Thr-Asn-Thr-Gly-OH(24-28)	Fragment 21
Boc-Leu-Gln-Thr-Tyr-Pro-OH(19-23)	Fragment 31
Boc-Leu-His-Lys(Psc)-OH(16-18)	Fragment 41
Boc-Lys(Psc)-Leu-Ser-Gln-Glu(OPse)-OH(11-15)	Fragment 51
H-Ser-Thr-Cys(Acm)-Val-Leu-Gly-ONse TFA(5-10)	Fragment 61
Boc-Cys(Acm)-Ser-Asn-Leu-OH(1-4)	Fragment 71

According to the invention, fragments 11 to 71 used in the synthesis of

salmon calcitonin by the segment condensation are prepared by the use of only Boc group which is common, inexpensive, and simple to blocking and deblocking, differing from the other methodologies (example: JP 6-16694A) using various groups for the temporary masking of α-amino groups. Size and number of the fragments are optimized to reduce the susceptibility of racemization; firstly, glycine or proline are selected as C-terminal residue of segments. In particular, new side chain protected amino acids are introduced to synthesize the fragments for the preparation of larger quantities and higher qualities of salmon calcitonin. The procedure for preparing salmon calcitonin by segment condensation based on the invention is represented in the followig diagram 1.

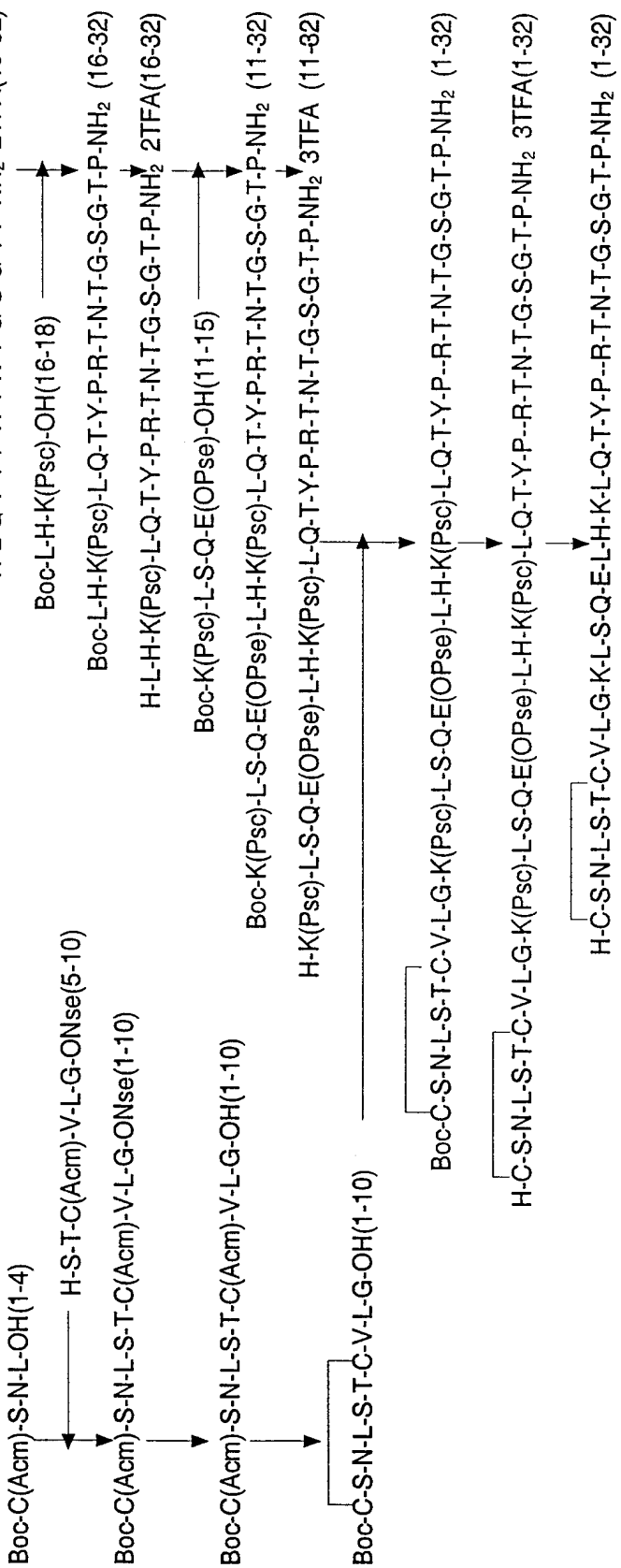

H-S-G-T-P-NH2TFA(29-32)
Boc-R-T-N-T-G-OH(24-28)
Boc-R-T-N-T-G-S-G-T-P-NH2(24-32)
H-R-T-N-T-G-S-G-T-P-NH2 2TFA(24-32)
Boc-L-Q-T-Y-P-OH(19-23)
Boc-L-Q-T-Y-P-R-T-N-T-G-S-G-T-P-NH2(19-32)
H-L-Q-T-Y-P-R-T-N-T-G-S-G-T-P-NH2 2TFA(19-32)
Boc-L-H-K(Psc)-OH(16-18)
Boc-L-H-K(Psc)-L-Q-T-Y-P-R-T-N-T-G-S-G-T-P-NH2 (16-32)
H-L-H-K(Psc)-L-Q-T-Y-P-R-T-N-T-G-S-G-T-P-NH2 2TFA(16-32)
Boc-K(Psc)-L-S-Q-E(OPse)-OH(11-15)
Boc-K(Psc)-L-S-Q-E(OPse)-L-H-K(Psc)-L-Q-T-Y-P-R-T-N-T-G-S-G-T-P-NH2 (11-32)
H-K(Psc)-L-S-Q-E(OPse)-L-H-K(Psc)-L-Q-T-Y-P-R-T-N-T-G-S-G-T-P-NH2 3TFA (11-32)
Boc-C(Acm)-S-N-L-OH(1-4)
H-S-T-C(Acm)-V-L-G-ONse(5-10)
Boc-C(Acm)-S-N-L-S-T-C(Acm)-V-L-G-ONse(1-10)
Boc-C(Acm)-S-N-L-S-T-C(Acm)-V-L-G-OH(1-10)
Boc-C-S-N-L-S-T-C-V-L-G-OH(1-10)
Boc-C-S-N-L-S-T-C-V-L-G-K(Psc)-L-S-Q-E(OPse)-L-H-K(Psc)-L-Q-T-Y-P--R-T-N-T-G-S-G-T-P-NH2 (1-32)
H-C-S-N-L-S-T-C-V-L-G-K(Psc)-L-S-Q-E(OPse)-L-H-K(Psc)-L-Q-T-Y-P--R-T-N-T-G-S-G-T-P-NH2 3TFA(1-32)
H-C-S-N-L-S-T-C-V-L-G-K-L-S-Q-E-L-H-K-L-Q-T-Y-P--R-T-N-T-G-S-G-T-P-NH2 (1-32)

Diagram 1

In the cases of lysine, glutamic acid, arginine, histidine, asparagine, etc., active side chains have to be protected to prevent blanching the chain in the peptide synthesis, generally. And the blocking group applied for this purpose should be unaffected during the coupling step and the removal of α-amino protecting group, and deblocked usually only at the end of the chain building process.

Selection of the side chain protecting group plays important role in the design for the synthethic route of salmon calcitonin, since it contains glutamic acid with side carboxyl group and lysine with side amino group which have to be protected semipermanently till the completion of peptide chain. A classical approach, the use of t-butyl or benzyl group for the protection of side carboxyl function of glutamic acid and of benzyloxycarbonyl(Z) or t-butyloxycarbonyl(Boc) or trityl(Trt) group for the protection of side amino function, is still practiced mainly. Yet, there are certain limitations inherent in the groups; the methods for deblocking, catalytic hydrogenation and acidolysis, have susceptibility of side reaction and are time consuming.

To solve the problems, the invention is intended to develope new base-labile side chain protecting group which is easy to introduce, stable during coupling, and readily removed under mild and selective conditions in combination with Boc used for the transient protection of the α-amino function. As a result, Pse and Psc groups are exploited to the protection of the side chain of glutamic acid and lysine, respectively. These groups without negative inductive effect from para substituent of phenyl group are stable not only under acidolysis and catalytic hydrogenation, but also under weak basic coupling condition, nevertheless these are readily removed by addition of piperidine within shorter time. These group protected amino acid derivatives, namely, Boc-Lys(Psc)-OH DCHA(**5**), Boc-Asp(OPse)-OH DCHA(**8**), Boc-Glu(OPse)-OH DCHA(**9**) are newly introduced to the synthesis of calcitonin. These compounds are prepared by the procedure as shown in diagram 2; ε-amino function of lysine is protected by the addition of

phenylthioethylchloroformate(3) whilst the α-groups are chelated to copper(II), followed by the oxidation. β- and γ-carboxyl function of aspartic and glutamic acid are blocked by the addition of phenylthioethanol, followed by oxidation.

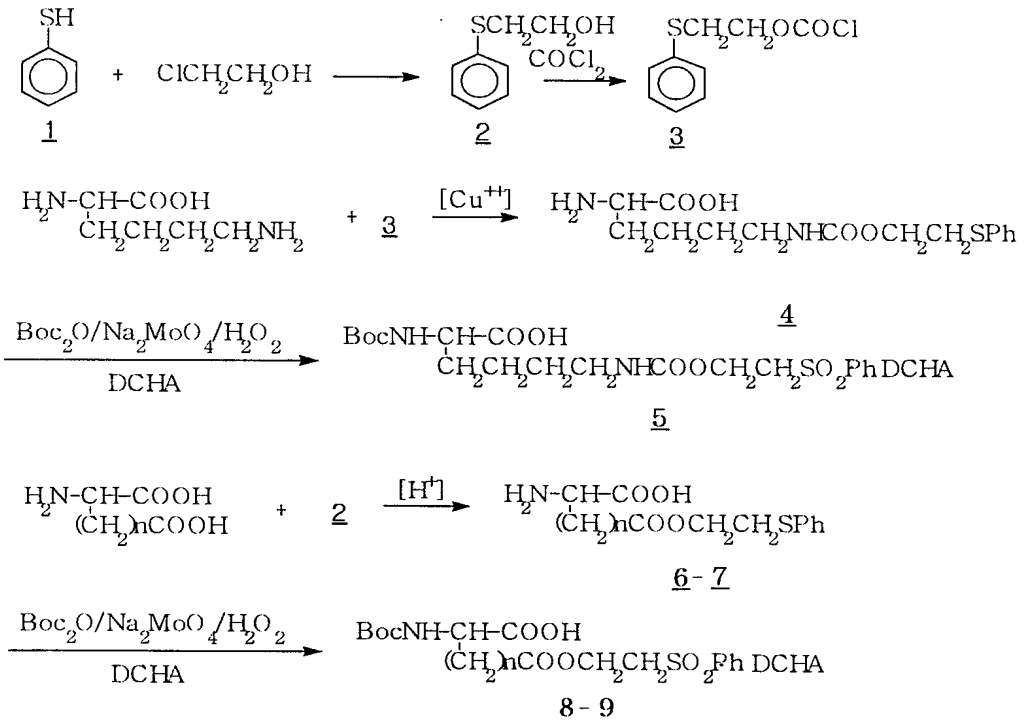


Diagram 2.

According to the invention, p-nitrophenylsulphonylethyl(Nse) group is newly employed as the masking group of C-terminal of fragment 6 and 7. In selecting the blocking group of C-terminal, It should be considered that the group is stable under coupling and removal of the α-amino protecting group, and readily removable under mild condition, and orthogonal with the side chain protecting group, too. So, it is intended to utilize the Nse group known in the art(Samukov, V. V. *Tetrahedron Lett.* 35 **1994**, 7821 : US pat. 5,527,881) for protecting C-terminal, which is selectively removal by base, and stable under the acidolysis and the catalytic hydrogenation.

The invention will now be illustrated by means of the following non limiting examples.

EXAMPLE

All the amino acids in the following description have L-configuration unless otherwise indicated. Melting points were taken on a Buch apparatus. Optical rotations were measured on a Jasco DIP 1000. HPLC: Hewlett pakard 1100. FT-IR: Jasco 300F. Atomic Absorption Spectroscopy(AAS): Shimadzu AA-6601F. UV: Shimadzu UV-265. TLC was done on silicagel 60 F-254 precoated plates(Merck); eluents: 1)ethyl acetate(EtOAc)/pyridine/acetic acid(AcOH)/water=42/14/6.6/1 2)n-BuOH/water/AcOH=4/1/1 3)acetonitrile(AcCN)/AcOH/water=8/0.2/2 4)AcCN/water/TFA/AcOH=20/5/0.1 5)$CHCl_3$/MeOH/AcOH=95/5/3 6)$CHCl_3$/MeOH/AcOH=90/10/3.

The abbreviations used in the description have the following meanings;

Acm: acetamidomethyl

Boc: t-buthyloxycarbonyl

Bz: benzyl

DCC: dicyclohexylcarbodiimide

DCHA: dicyclohexylamine

DMAP: dimethylaminopyridine

DMF: N,N'-dimethylformamide

EDTA: ethylenediaminetetraacetic acid

Et: ethyl

HOBT: hydroxybenzotriazole

NMM: N-methyl morpholine

Nse: p-nitrophenylsulphonylethyl

OPse: phenylsulphonylethyloxy

OPte: phenylthioethyloxy

pfp: pentafluorophenyl

Psc: phenylsulphonylethyloxycarbonyl

Pse: phenylsulphonylethyl

Ptc: phenylthioethyloxycarbonyl

Pte: phenylthioethyl

Su: succinimide

Tce: trichloroethyl

Tcp: trichlorophenyl

TEA: triethyl amine

TFA: trifluoroacetic acid

C: cysteine E: glutamic acid G: glysine H: histidine K: lysine L: leucine N: asparagine P: proline Q: glutamine R: arginine S: serine T: threonine V: valine Y: tyrosine

Example 1. preparation of amino acid derivatives

a) H-Lys(Ptc)-OH(4)

A suspension of 20.1g of lysine hydrochloric acid salt and 12g of copper basic carbonate in 250mL of water was boiled for 30min, filtered, and washed with 30mL of water. To the filtrate 125mL of dioxane and 55mL of 2N sodium hydroxide were added, and cooled in ice bath. Then the solution of 21.7g of 2-phenylthioethyl chloroformate in 50mL of dioxane and 50mL of 2N sodium hydroxide were added to the mixture dropwise within 1.5 hr with stirring. After additional stirring for 2 hr, the mixture was filtered, and washed with 150mL of water, 150mL of acetone, and 50mL of ether, successively. Collected precipitat was dissolved in 500mL of 2N hydrochloric acid. To the mixture a suspention of 30g of EDTA in 1L of water was added with stirring. Then the mixture was acidified to pH 4, washed with water, and dried on air to give 26g(77%) of the desired compound **4** as white powder.

m.p.: 255-259°C

TLC: Rf=0.65 (eluent 3)

$[\alpha]_D$: +12.9 (20°C, 0.5N HCl)

HPLC: purity 100%, Rt=19.5min

IR(KBr, cm^{-1}): 3343, 2947, 1683, 1578, 1533, 1418, 1325, 1236, 734

b) Boc-Lys(Psc)-OH(5)

To the mixture of 19.6g of **4**, 12.5g of potassium carbonate, and 350mL of

water-i-propanol-DMF mixture(4:2:1, v/v), 16mL of Boc_2O was added with stirring at 45-50°, and stirred for additional 3 hr. The mixture was concentrated to 1/2 of volume, diluted with 500mL of water, and washed with 150mL of ether. 200mL of EtOAc was added to aqueous layer, followed by acidification to pH=1.5. More 150ml of EtOAc was added, and organic layer was seperated from the mixture, and washed with 150mL of water and 100mL of brine, and dried under anhydrous Na_2SO_4. Evaporation of the filtrate gave 26g of oil. The oil was dissolved in 300mL of acetone. And 15mL of 0.3M sodium molibdate and 14mL of hydrogen peroxide were added to the solution. After 1 hr, the mixture was stirred for 5 hr at 50°C, and evaporated. The residue was diluted with 300mL of water and 300mL of EtOAc, and organic layer was separated, and washed with 150mL of water, 150mL of 0.5N hydrochloric acid, and 100mL of brine, successively. Then the solution was dried unser anhydrous sodium sulfate, evaporated, and solidified by addition of DCHA. The precipitate formed was filtered, and dried *in vacuo* to give 36.5g(95%) of **5** as white powder.

m.p.: 109-112°C

TLC: Rf=0.40 (eluent 5)

$[\alpha]_D$: +7.2 (20°C, 10% AcOH)

HPLC: purity 100%, Rt=21.4min

IR(KBr, cm^{-1}): 3393, 2933, 2858, 1698, 1636, 1560, 1398, 1318, 1254, 1149, 1051, 728,

c) H-Asp(OPte)-OH(6)

13.3g of aspartic acid was dissolved in 90mL of dimethoxyethane, then 10mL of concentrated sulfuric acid and 40mL of 2-phenylthioethanol were added to the mixture. The mixture was stirred until clearness, then left for 2 day at room temperature. After evaporation a solution of 30g of sodium acetate in 300mL of water was added to the residue with vigorously stirring. White precipitate formed was filtered, washed, and dried on air to afford 14.6g(54%) of **6**

m.p.: 215-217°C

TLC: Rf=0.80 (eluent 2)

$[\alpha]_D$: +21.2 (22°C, 1N HCl)

HPLC: purity 100%, Rt=17.6min

IR(KBr, cm^{-1}): 3123, 2379, 2306, 1739, 1636, 1416, 1343, 1135, 730, 688

d) Boc-Asp(OPse)-OH DCHA(8)

To the mixture of 14.0g of **6**, 8.4mL of TEA, and 50mL of DMF, 15mL of Boc_2O was added with stirring at 45-50°, and stirred for additional 3 hr. The mixture was diluted with 500mL of water, and extracted with 500mL of 5% potassium hydrogen sulfate. The organic layer was washed with 2 X 250mL of 5% potassium hydrogen sulfate and 200mL of brine, and dried under anhydrous sodium sulfate. After filtration, the filtrate was evaporated to oil. The oil was dissolved in 250mL of acetone. And 13mL of 0.3M sodium molibdate and 12mL of hydrogen peroxide were added to the solution. After 1 hr, the mixture was stirred for 5 hr at 50°C, and evaporated. The residue was diluted with 250mL of water and 250mL of EtOAc, and organic layer was separated, and washed with 150mL of water, 150mL of 0.5N hydrochloric acid, and finally 100mL of brine. Then the solution was dried under anhydrous sodium sulfate, evaporated, and solidified by adding DCHA. The precipitate formed was filtered, and dried *in vacuo* to give 28.8g(95%) of **8** as white powder.

m.p.: 142-144°C

TLC: Rf=0.20 (eluent 5)

$[\alpha]_D$: -5.4 (23°C, 10% AcOH)

HPLC: purity 100%, Rt=26.5min

IR (KBr, cm^{-1}): 3397, 2937, 2866, 1740, 1708, 1584, 1489, 1397, 1318, 1149

e) H-Glu(OPte)-OH(7)

The compound **7** as white powder was obtained by the same procedure as described in c) with an yield of 55%.

m.p.: 182-183°C

TLC: Rf=0.55 (eluent 2)

$[\alpha]_D$: +17.65 (20°C, 0.5N HCl)

HPLC: purity 100%, Rt=18.7min

IR(KBr, cm^{-1}): 2955, 1726, 1586, 1508, 1421, 1202, 733

f) Boc-Glu(OPse)-OH DCHA(9)

The compound **9** as white powder was prepared by the same procedure as described in d) with an yield of 92%.

m.p.: 155-157°C

TLC: Rf=0.36 (eluent 5)

$[\alpha]_D$: -5.8 (20°C, 10% AcOH)

HPLC: purity 100%, Rt=21.5min

IR(KBr, cm^{-1}): 2938, 2857, 1733, 1701, 1637, 1560, 1449, 1399, 1297, 1141, 1085, 727, 686

Example 2 Synthesis of salmon calcitonin

a)H-Ser-Gly-Thr-Pro-NH_2(fragment 11, 10)

Fragment 11(purity>99%, Rt=6.54min: HPLC) as white powder was obtained by the conventional stepwise coupling using active esters with an overall yield of 75%

reactant 1	reactant 2	coupling/ debloking	product	yield	Rf
Boc-Thr-OPfp 17.0g	H-Pro-NH_2 HCl 6.2g	active ester/TFA	H-T-P-NH_2 TFA **17**	93% (12.4g)	1)0.13
Boc-Gly-OPfp 13.6g	**17** 12.0g	active ester/TFA	H-G-T-P-NH_2 TFA **18**	95% (14.0g)	1)0.09
Boc-Ser-OPfp 14.8g	**18** 14.0g	active ester/TFA	H-S-G-T-P-NH_2 TFA **10**	85% (13.0g)	2)0.17

b) Boc-Arg-Thr-Asn-Thr-Gly-OH(fragment 21, 11)

Fragment 21(purity>99%, Rt=12.6min : HPLC) as white powder was obtained by the stepwise synthesis using acive esters or condensing agents(DCC, HOBT) well known, followed by catalytic(Pd-C) reduction for deblocking of Benzyl group with an overall yield of 59%.

reactant1	reactant 2	coupling/ debloking	product	yield	Rf
Boc-Thr-OH 9.6g	H-Gly-OBz TsOH 13.6g	DCC, HOBT/ TFA	H-T-G-OBz TFA **19**	90% (14.0g)	1)0.32
Boc-Asn-OH 9.4g	**19** 13.8g	DCC, HOBT/ TFA	H-N-T-G-OBz TFA **20**	86% (15.6g)	1)0.17
Boc-Thr-OPfp 12.4g	**20** 14.6g	active ester/TFA	H-T-N-T-G-OBz TFA **21**	90% (15.6g)	1)0.20
Boc-Arg-OH 8.2g	**21** 15.6g	active ester	Boc-R-T-N-T-G-OBz TFA **22**	100% (22.6g)	1)0.40
	22 22.6g	Pd-C, H_2	Boc-R-T-N-T-G-OH TFA **11**	84% (14.4g)	1)0.17

c) Boc-Leu-Gln-Thr-Tyr-Pro-OH(fragment 31, 12)

Fragment 31(purity>99%, Rt=18.9min: HPLC) as white powder was obtained by the conventional stepwise coupling using condensing agents(DCC, HOBT), followed by base catalized hydrolysis and catalytic(Pd-C) reduction with an overall yield of 49%.

reactant1	reactant2	coupling/ debloking	product	yield	Rf
Boc-Tyr(Bz)-OH 15.0g	H-Pro-OEt 9.0g	DCC,HOBT/ TFA	H-Y(Bz)-P-OEt TFA **23**	quantitative (26.0g)	1)0.10
Boc-Thr-OH 9.2g	**23** 26.0g	DCC,HOBT/ TFA	H-T-Y(Bz)-P-OEt TFA **24**	quantitative (30.0g)	1)0.10
Boc-Gln-OH 9.8g	**24** 30.0g	DCC,HOBT/ TFA	H-Q-T-Y(Bz)-P-OEt TFA **25**	70% (21.0g)	1)0.37
Boc-Leu-OH 10.0g	**25** 21.0g	DCC,HOBT/ TFA	Boc-L-Q-T-Y(Bz)-P-OEt **26**	85% (20.4g)	1)0.37
	26 20.4g	NaOH, water, MeOH	Boc-L-Q-T-Y(Bz)-P-OH **27**	90% (17.6g)	5)0.27
	27 17.6g	Pd-C, H_2	Boc-L-Q-T-Y-P-OH **12**	92% (14.6g)	3)0.58

d) Boc-Leu-His-Lys(Psc)-OH(fragment 41, 13)

Fragment 41 was obtained by the following steps with an overall yield of 81%; firstly, α-carboxyl function of Boc-Lys(Psc)-OH was blocked by trichloroethyl group, and coupled stepwisely after deblocking of Boc, finally deprotected by catalytic(Zn) hydrogenation to give **13** (purity>99%, Rt=23.6min: HPLC) as white powder.

reactant 1	reactant 2	coupling/ debloking	product	yield	Rf
Boc-His(Boc)-OTcp 10.2g	H-Lys(Psc)-OTce 10.5g	active ester	Boc-H-K(Psc)-OTce **28**	94% (15.5g)	4)0.63
Boc-Leu-OSu 8.7g	**28** 15.5g	active ester /HCl,AcOH	Boc-L-H-K(Psc)-OTce **29**	93% (14.6g)	5)0.33
	29 14.6g	Zn, AcOH	Boc-L-H-K(Psc)-OH **13**	93% (11.5g)	1)0.30

e) Boc-Lys(Psc)-Leu-Ser-Gln-Glu(OPse)-OH(fragment 51, 14)

Fragment 51(purity>99%, Rt=23.5min: HPLC) was obtained by the conventional stepwise coupling as follows with an overall yield of 58%; firstly, α-carboxyl function of Boc-Glu(OPse)-OH was blocked by

trichloroethyl group, and coupled stepwisely by the methods as shown below after deblocking of Boc, finally deprotected by catalytic hydrogenation to give **13** as white powder.

reactant 1	reactant 2	coupling/ debloking	product	yield	Rf
Boc-Gln-OH 7.4g	H-Glu(OPse) -OTce TFA 14.5g	DCC,HOBT	Boc-Q-E(OPse)-OTce **30**	93% (16.3g)	5)0.42
Boc-Ser-OPfp 10.0g	**30** 16.3g	TFA/ DCC,HOBT	Boc-S-Q-E(OPse)-OTce **31**	90% (16.5g)	5)0.32
Boc-Leu-OTcp 10.3g	**31** 16.5g	TFA/ active ester	Boc-L-S-Q-E(OPse)-OTce **32**	88% (16.7g)	5)0.42
Boc-Lys(Psc)-OTcp 13.8g	**32** 16.7g	TFA/ active ester	Boc-K(Psc)-L-S-Q-E(OPse)-OTce **33**	93% (21.5g)	5)0.33
	33 21.5g	Zn, AcOH	Boc-K(Psc)-L-S-Q-E(OPse)-OH **14**	85% (16.0g)	5)0.22

f) H-Ser-Thr-Cys(Acm)-Val-Leu-Gly-ONse TFA(fragment 61, 15)

Fragment 61(purity>99%, Rt=19.6min : HPLC) as white powder was obtained by the procedure as shown below with an overall yield of 67%; α -carboxyl function of Boc-Gly-OH was blocked by Nse group, and then after deblocking of Boc coupled stepwisely by the conventional methods using active esters or condensing agents(DCC, HOBT) to give **15** as white powder.

reactant 1	reactant 2	coupling/ debloking	product	yield	Rf
Boc-Leu-OH 5.9g	H-Gly-ONse 8.3g	DCC,HOBT/ TFA	H-L-G-ONse **34**	94% (10.1g)	1)0.47
Boc-Val-OH 5.2g	**34** 10.1g	DCC,HOBT/ TFA	H-V-L-G-ONse **35**	89% (10.6g)	1)0.48
Boc-Cys(Acm)-OPfp 5.9g	**35** 10.6g	active ester/TFA	H-C(Acm)-V-L-G-ONse **36**	94% (12.8g)	1)0.47
Boc-Thr-OPfp 7.1g	**36** 12.8g	active ester/TFA	H-T-C(Acm)-V-L-G-ONse **37**	98% (14.2g)	1)0.27
Boc-Ser-OPfp 5.9g	**37** 14.2g	active ester/TFA	H-S-T-C(Acm)-V-L-G-ONse **15**	87% (13.7g)	1)0.38

g) H-Cys(Acm)-Ser-Asn-Leu-OH(fragment 71, 16)

Fragment 71 was obtained by the procedure as described below; at first, α-carboxyl function of Boc-leu-OH was protected by the additon of Nse-OH in the presence of DMAP, then the Boc group was deprotected to afford H-Leu-ONse quantitatively. Then the product was stepwisely coupled by the conventional methods using active esters or condensing agents(DCC, HOBT), finally deprotected by piperidine to give **16**(purity>99%, Rt=16.4min: HPLC) as white powder.

reactant 1	reactant 2	coupling/ debloking	product	yield	Rf
Boc-Asn-OH 8.2g	H-Leu-ONse TFA 14.5g	DCC,HOBT/ TFA	H-N-L-ONse TFA **38**	85% (15.4g)	1)0.36
Boc-Ser-OPfp 11.1g	**38** 15.4g	active ester /TFA	H-S-N-L-ONse TFA **39**	94% (16.6g)	1)0.23
Boc-Cys(Acm)-OPfp 9.1g	**39** 16.6g	active ester	Boc-C(Acm)-S-N-L-ONse **40**	93% (19.1g)	5)0.43
	40 19.1g	piperidine	Boc-C(Acm)-S-N-L-OH **16**	87% (12.3g)	2)0.57

h)H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2 (salmon calcitonin)

Biological active salmon calcitonin as white powder was prepared by the segment condensation as shown in diagram 1. The synthesis was started from i) the condensation of fragment 1, the tetrapeptide corresponding to the carboxamide end of the salmon calcitonin sequence, with Boc-protected fragment 2 by the conventional method using DCC and HOBT. And stepwise condensations with Boc-protected fragment 3, 4, 5 and Boc group removals were mediated by the DCC/HOBT and TFA, respectively, to give newly side chain protected docosapeptide **45** in moderate yield. For the preparation of cyclic decapeptide **46** Acm-protected fragment 6 was condensed with Acm-protected fragment 7 by the conventional method, the Nse group for

C-terminal blocking was deprotected with piperidine, and two Acm groups for masking of thiol function were simultaneously deprotected/oxidated with iodine in AcOH. After the final condensation of docosapeptide **45** with decapeptide **46**, the resulting peptide was treated with TFA and piperidine in DMSO for shorter time to afford salmon calcitonin which showed a purity of 66% to 70% in analytical HPLC. The crude one was purified by preparative HPLC to give salmon calcitonin with high purity of above 98%.

m.p.: 220°C

$[\alpha]_D$: -55.0 (20°C, 50% AcOH)

λ_{max}: 275nm

LC/Mass: (1mm, C-18 column, 40μl/min, water/Acetonitrile/TFA buffer)

m/z 3431.8(M^+) 1717.2($[M+2H]^{2+}$) 1144.8($[M+3H]^{3+}$) 859.0($[M=4H]^{4+}$

Rt=22.90min

AAA: Arg(0.93) Asp(0.97) Cys/2(1.0) Glu(0.90) Gly(1.0) His(0.92) Leu(1.05) Lys(1.0) Pro(0.92) Ser(0.91) Thr(0.98) Tyr(1.1)

reactant 1	reactant 2	coupling/ debloking	product	yield	Rf
11 8.6g	**10** 10.5g	DCC,HOBT/ TFA	H-R-T-N-G-S-G-T-P-NH_2 2TFA **42**	83% (15.0g)	1)0.13
12 10.4g	**42** 14.3g	DCC,HOBT/ TFA	H-L-Q-T-Y-P-R-T-N-G-S-G-T-P -NH_2 2TFA **43**	79% (17.4g)	1)0.13
13 7.7g	**43** 17.0g	DCC,HOBT/ TFA	H-L-H-K(Psc)-L-Q-T-Y-P-R-T-N-G -S-G-T-P-NH_2 3TFA **44**	90% (21.5g)	4)3) 0.24
14 12.0g	**44** 20.1g	DCC,HOBT/ TFA	H-K(Psc)-L-S-Q-G(OPse)-L-H-K (Psc)-L-Q-T-Y-P-R-T-N-G-S-G-T -P-NH_2 3TFA **45**	75% (21.2g)	6)0.38
15 13.6g	**16** 10.6g	DCC,HOBT/ i)piperidin ii)I_2,AcOH	Boc-C-S-N-L-S-T-C-V-L-G-OH (1,7-disulfide bond) **46**	77% (12.0g)	4)3) 0.32
45 20g	**46** 10.0g	DCC,HOBT/ i)TFA ii)piperidin	salmon calcitonin	74% (18.0g)	

Example 3. Biological assay of salmon calcitonin

Hypocalcaemic effect of salmon calcitonin was measured in rats by comparing with standard(NIBSC: National Institute for Biological Standards and Control). 30 rats of the same sex weighing up to 225g were divided into 6 groups. After deprive for 1 day, 3 groups are injected by standard preparation(1,3,9mIU/0.25mL of albumin solution, rat/ 100g of body weight), the other groups synthetic one. Exactly 1 hr after injection, a sample of blood was taken, the plasma of it was separated from cells. Then the calcium content of each sample was determined by atomic absorption spectroscopy, and the relationship between the calcium concentration and the logarithm of the dose was calculated by standard statistical methods.

subcutaneously injected concentration (mIU / 100g body weight)	biological activity compared with standard preparation
9	99.75 %
3	98.65 %
1	98.75 %

It is demonstrated that according to this invention, target peptide can be prepared by using new amino acid derivatives which are very stable under coupling and deblocking with good yield and in high purity, which shows high bilogical activity.

Claims

1. A process for preparing the peptides as shown in general formula (II) by means of stepwise condensation with the following fragments 1 to 7.

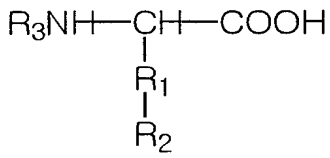
H-Cys-A^1-A^2-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-A^3-A^4-Thr-Gly-A^5-Gly-Thr-Pro-NH_2

(II)

H-A^5-Gly-Thr-Pro-NH_2 (29-32)	Fragment 1
Boc-Arg-A^3-A^4-Thr-Gly-OH (24-28)	Fragment 2
Boc-Leu-Gln-Thr-Tyr-Pro-OH (19-23)	Fragment 3
Boc-Leu-His-Lys(X^1)-OH (16-18)	Fragment 4
Boc-Lys(X^1)-Leu-Ser-Gln-Glu(X^2)-OH (11-15)	Fragment 5
H-Ser-Thr-Cys(X^3)-Val-Leu-Gly-OY TFA (5-10)	Fragment 6
Boc-Cys(X^3)-A^1-A^2-Leu-OH(1-4)	Fragment 7

wherein A^1 may represents Ala or Ser, and A^2 may represents Asn or Ser, and A^3 may represents Asn or Asp(OPse)(wherein Pse is phenylsulfonylethyl), and A^4 may represents Thr or Val, and A^5 may represents Ala or Ser; X^1 may represents phenylsulfonylethoxycarbonyl(Psc), benzyloxycarbonyl(Z), or fluorenylmethoxycarbonyl(Fmoc) group for blocking of the side amino function of lysine, and X^2 may represents phenylsulfonylethyl(Pse), t-butyl, benzyl, or p-nitrobenzyl group for blocking of the side carboxyl function of glutamic acid, and X^3 may represents acetamidomethyl(Acm) or trityl(Trt) group for blocking of the thiol function of cysteine; Y may represents p-nitrophenylsulfonylethyl(Nse), phenylsulfonylethyl(Pse), benzyl or ethyl group for blocking of C-terminal.

2. A process according to claim 1, wherein the peptide fragments are obtained by using a side chain protected amino acid derivative of the general formula (I) or pharmaceutically acceptable salt thereof.



(I)

wherein R^1 may represents $-CH_2CH_2CH_2CH_2NH-$, $-CH_2CO-$ or $-CH_2CH_2CO-$, and R^2 may represents phenylthioethyloxycarbonyl(Ptc), phenylsulfonylethyloxycarbonyl(Psc), phenylthioethyloxy(OPte) or phenylsulfonylethyloxy(OPse), and R^3 may represents hydrogen or α-amino protecting group.

3. A process according to claim 1 or 2, wherein the peptide fragments are obtained by using Boc-Lys(Psc)-OH, Boc-Glu(OPse)-OH, Boc-Asp(OPse)-OH or pharmaceutically acceptable salt thereof.

4. A process according to claim 1, wherein only Boc group is used for the protection of α-amino function.

5. A process according to claim 1, wherein Nse group is used for the protection of α-carboxyl function.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00173

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 07 K 14/585 // A 61 K 38/23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: A 61 K; C 07 K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Database WPI on EPOQUE, week 9542, London: Derwent Publications Ltd., AN 95-322942, Class B04, JP 7188297 A (DAICEL CHEM. IND., LTD.), abstract.	1-5
A	WO 97/29 127 A1 (BIONEBRASKA, INC.) 14 August 1997 (14.08.97), claim 21.	1-5

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 97/00173

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9729127	14-08-97	AU A1 22545/97	28-08-97